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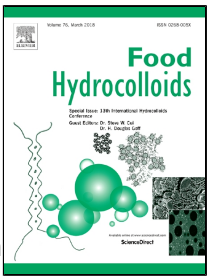

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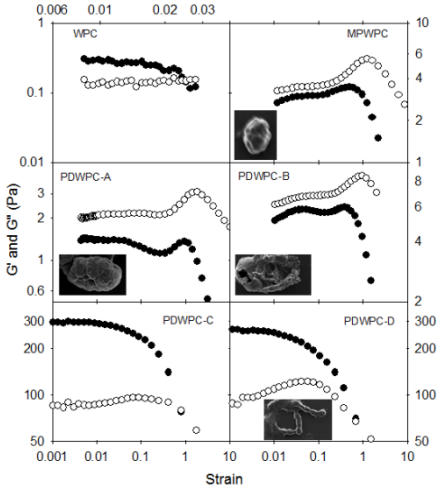
Graphical Abstract



Properties of Partially Denatured Whey Protein Products: Viscoelastic Properties

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Abstract

Partially denatured whey protein products (PDWPC's) can be classified based on the viscoelastic properties of their solutions. Strain sweeps show that PDWPC-A and –B and microparticulated WPC (MPWPC) with compact, spherical aggregated particles exhibit a strong strain overshoot. PDWPC-C and –D, on the other hand, which have open, elongated porous particles show a weak strain overshoot. The concentration dependence of the elastic modulus G' in the linear viscoelastic region has a biphasic power law dependence with concentration for all protein products studied, except for WPC where G' is independent of protein concentration. Frequency sweeps suggest that MPWC solutions form a strong physical gel at all concentrations above 14% (w/w). PDWPC-A and –B form weak gels over the same concentration range. PDWPC-C and –D also form weak gels at 14% protein (w/w) but strong physical gels at higher concentrations. The frequency dependence of G' and G'' for all aggregated proteins show a power law dependence indicating fractal type structures. For all solutions above a critical concentration, the fractal dimensions span the range 1.6-2.3, indicating a range of gel network structures from open and diffuse to compact and dense. Adherence to the empirical Cox-Merz rule was observed in PDWPC-A, -C and –D at concentrations of 14 and 16% (w/w) protein, suggesting liquid-like behaviour. At higher protein concentrations the deviations from the Cox-Merz rule suggest more pronounced elasticity in the structure. For PDWPC-B, the behaviour is

complex, with deviation from the Cox-Merz rule at low frequencies/shear rates, but correspondence at higher frequencies/shear rates at all concentrations. This indicates a frequency-dependent change from liquid-like behaviour over long timescale deformations, to a solid-like behaviour at short timescale deformations. MPWPC solutions of all concentrations do not follow the Cox-Merz rule, suggesting solid-like behaviour. The PDWPCs exhibit a complex rheological behaviour which suggests they could be versatile thickening, texturizing and fat replacement ingredients that will offer food manufacturers greater control over the texture of formulated fluid foods.

Keywords: Partially denatured whey proteins; oscillatory rheology; frequency sweep; fractal; Cox-Merz rule

Introduction

Heat-processed whey proteins have been investigated as thickening and gelling ingredients in foods, and in particular as potential fat replacers (McClements, Chung & Wu, 2017). It has been widely reported that whey proteins that are partially denatured with pre-heat treatment at low ionic strength exhibit gelling properties at room temperature and thus are defined as cold-gelling whey protein concentrates (WPC's) (McClements & Keogh, 1995). The cold-setting gelation could be of great value in the food industry, and would meet the thickening requirement for some special foods that are not suitable for heat processing.

In previous papers (Zhang, Arrighi, Campbell, Lonchamp &. Euston, 2016a; 2016b) we reported on the manufacture of partially denatured whey protein products (PDWPCs) that exhibit differing viscosity modifying properties dependent on the method used for processing. More specifically, we showed that by controlling the degree of disulphide bonding between aggregates (by altering the temperature, pH and heating time) it was possible to form PDWPC particles of differing degree of protein denaturation and particle size and morphology (Zhang et al., 2016a). All PDWPCs gave solutions of increased viscosity compared to the starting whey protein concentrate solution (Zhang et al., 2016b). Particles with a high degree of denaturation and an open phase separated morphology gave much higher viscosities than particles of a lower

degree of denaturation that had a more compact globule-like structure (Zhang et al., 2016a). These differences in viscosity are believed to arise through differences in the way the particles interact with the water hydration shell around the proteins, and the interactions between the particles themselves. A detailed study of the flow properties of PDWPCs revealed a complex dependence of the shear viscosity on the particle morphology, protein concentration in solution and shear rate (Zhang et al., 2016b). The surface structure of protein particles also plays a role, with particles of similar size but with a rougher surface giving a higher viscosity for the same protein concentration. These studies indicated that by controlling the aggregated whey protein particle structure through changes in processing conditions, it is possible to make protein products with a range of viscosity modifying potential that could find application in food systems. To understand better the rheology of PDWPCs we have extended our studies to look at the viscoelastic properties of their solutions, and in particular strain and frequency dependent properties.

4 Material and methods

Protein solutions

Commercial protein products that we have characterised previously (Zhang et al., 2016a) were used for these studies. A whey protein concentrate (WPC) Lacprodan 87 (Arla Foods Ingredients, Denmark), a microparticulated WPC

(MPWPC) (Simplesse® 100[E], CP Kelco UK Limited, UK) and a series of partially denatured whey protein (PDWPC) products, labelled PDWPC-A, PDWPC-B, PDWPC-C, and PDWPC-D (Nandi Proteins, UK) were dissolved in deionized distilled water at room temperature to make solutions with protein concentrations of 6%, 9%, 12%, 14%, 16%, 18% and 21% (w/w). The solutions were stirred gently for at least 1 h to allow hydration of the proteins. Previous results (Zhang et al., 2016b) have shown that hydration (as assessed by change in the constant shear viscosity with time) is complete in under an hour for all protein particles. The pH of all solutions was in the range 6.7-7.0 and the pH was not adjusted before testing. The same procedure was repeated for a further set of 21% (w/w) protein solutions, except that acetic acid (≥99.7 w/w %) (Fisher Scientific, USA) was added to adjust the pH to 4.5. The processing conditions used to prepare the PDWPC's and their properties have been reported by us elsewhere (Zhang et al., 2016a). Briefly, the PDWPC's were processed at differing temperature/pH combinations, 73 ^{o}C/pH 6.5, 72.5 ^{o}C/pH 6.4, 72.5 ^{o}C/pH 7.0, 74 ^{o}C/pH 7.0 giving a degree of denaturation of 65, 45, 51 and 98% for PDWPC-A, -B, -C and –D respectively.

Oscillatory rheology

Oscillation measurements were performed with controlled stress mode using a Bohlin Gemini rheometer (Malvern Instruments, UK), with 4°/40 mm cone and plate at a temperature of 20 °C. Prior to oscillatory testing, the linear

viscoelastic region (LVER) was found by amplitude sweep measurements taken at a frequency of 1 Hz. A stress/strain combination in the LVER was chosen to use for the frequency sweep measurements to ensure that during these tests the stress and strain was maintained below that which would disrupt the solid structure of the solutions.

Frequency sweep measurements were performed in the range from 0.001 Hz to 15 Hz, so that the largest angular frequency, ω, is of the order of 100 rad/s. Oscillatory measurements in the linear viscoelastic region are often employed for the investigation of gel systems, since the solid and liquid responses to shear stress can be distinguished (Clark, Kavanagh, & Ross-Murphy, 2001; Ikeda & Nishinari, 2001a). Comparing the storage (G') and loss (G'') moduli with each other allows to define a system as having solid-like ($G'>G''$), liquid-like ($G'<G''$), or critical gel ($G'=G''$) behaviour (Barnes, 2000; Barnes, Hutton, & Walters, 1989; Goodwin & Hughes, 2008; Macosko, 1994; Morrison, 2001). Moreover, the relationship between the dynamic moduli (G', G'') and the observation time, (represented experimentally as the frequency, ω, of oscillation) reveals the structure of the gels. Vilgis and Winter (1988), proposed a power law relationship between G'/G'' and ω for self-similar or fractal structures for chemical gels, which has been proven to be valid for physical gels (Miyoshi & Nishinari, 1999).

5 Results and discussion

To obtain the dynamic viscoelastic properties, the linear viscoelastic region of a material must be defined first, i.e. where linearity between strain and stress is observed (Barnes, 2000; Barnes et al., 1989; Goodwin & Hughes, 2008; Macosko, 1994; Morrison, 2001). Experimentally, this linear viscoelastic range is obtained from strain sweep measurements (Ikeda & Nishinari, 2001a). Representative strain sweeps at 21% protein content are plotted for all protein products in Figure 1. For WPC solutions of all concentrations the strain sweep data (not shown for protein concentrations 6-18%) an LVER was observed for all protein concentrations at low strains (below strain 0.02) in agreement with results of other researchers (Ikeda & Nishinari, 2000; 2001a). The G' was always higher than G'' in these LVER regions, suggesting significant protein-protein interaction and solid-like behaviour in this region. Ikeda & Nishinari (2000; 2001a) explain this in terms of a colloidal crystal-like behaviour where the proteins interact through long-range electrostatic interaction to form an ordered structure. In a previous study on the concentration dependence of shear viscosity in PDWPC solutions (Zhang et al., 2016b) we observed constant shear viscosity at low shear rates that was independent of WPC concentration. We interpreted this as evidence of ordered, solid-like colloidal crystal phases. These have been observed by others (Matsumoto & Inoue, 1996; Ikeda & Nishinari, 2000; Ikeda & Nishinari, 2001a; Ikeda & Nishinari, 2001b) for protein solutions at low shear rates, and have been attributed to

159 long-range, but weak repulsive electrostatic interactions between proteins.
160 Hyun et al. (Hyun, Kim, Ahn & Lee, 2002) have defined at least four types of
161 large amplitude oscillatory strain (LAOS) behaviour which depend on the
162 interactions between the microstructure elements in the solution. These are
163 type I, strain thinning (both G' and G'' decreasing with increasing strain); type
164 II, strain hardening (both G' and G'' increasing); type III, weak strain overshoot
165 (G' decreasing, G'' first increases followed by a decrease); type IV, strong
166 strain overshoot (both G' and G'' increase followed by a decrease). The type of
167 strain sweep behaviour seen for the WPC solutions is the so-called type I
168 behaviour as described by Hyun et al. (2002). In type I behaviour there is an
169 LVER region, followed by decreasing G' above a critical strain (strain thinning
170 behaviour). Type I behaviour is common in polymer solutions and its physical
171 explanation is similar to that for shear-thinning behaviour in solutions, i.e. at
172 low shear rate/strain the generated stress is too low to disrupt the structure,
173 but as the shear rate/strain increases proteins align along the flow direction.
174 This type of strain sweep behaviour is illustrated in Figure 1, where the strain
175 sweep for WPC at a concentration of 21% protein is plotted.
176 MPWPC and the PDWPC solutions show a different type of strain sweep
177 behaviour compared to WPC. The protein products show a strain thinning
178 behaviour at lower protein contents (9% and below for MPWPC, 18% and
179 below for PDWPC-A, and 14% and below for PDWPC-B, PDWPC-C and
180 PDWPC-D, data not shown). At higher protein contents, the strain sweep

behaviour changes to the type III or type IV behaviour described by Hyun et al. (2002). This type of behaviour is seen in Figure 1 where the strain sweep at 21% protein is plotted for all protein products. Type III behaviour, which is shown by PDWPC-C and PDWPC-D is characterised by a local maximum in the G" curve (Figure 1) above the LVER. This is found, typically, in systems such as concentrated emulsions (Bower, Gallegos, Mackley & Madiedo, 1999) and dispersed hydrogel spheres (Wyss et al., 2007). This type of behaviour, which is termed weak strain overshoot, is believed to be due to changes in the structure of flocs of particles under the action of the oscillatory shear stresses, although the exact mechanisms appear to depend on the nature of the system. Raghavan and Khan (1995) explain the appearance of weak strain overshoot as being due to an increase in the floc size at a critical stress/strain. Others (Tirtaatmadja, Tam & Jenkins, 1997) suggest this is because of an increase in the effective volume of the flocs. Parthasarathy & Klingenberg (1999) take a slightly different view and believe the increase in G" is due to small rearrangements of floc structure followed by larger rearrangements that lead to the decrease in G" again at larger strains. Type IV behaviour, or strong strain overshoot, is displayed by MPWPC, and PDWPC's A and B and is characterised by a maximum in both G" and G' (Hyun et al., 2002). This is believed to arise when there are relatively strong inter-particle interactions such as hydrophobic association. These results suggest that the MPWPC and PDWPC's A and B have stronger inter-particle interactions in solution

compared to PDWPC C and D, even though the plateau G' values are lower. These results agree with our earlier study of the shear viscosity behaviour of the protein products which showed that MPWPC, PDWPC-A and PDWPC-B have a strong time dependence for viscosity recovery from a step change in shear rate from 1 s^{-1} to 100 s^{-1} back to 1 s^{-1} (Zhang et al., 2016b), whereas PDWPC's C and D recover quickly. This again also indicates stronger interactions in the MPWPC and PDWPC's A and B that are slow to reform when high shear is removed. To explain the higher G' in the LVER and the higher viscosity for a given shear rate (Zhang et al., 2016b) for PDWPC's C and D, it should be noted that these have a much larger particle size than the other aggregated proteins, and for PDWPC-D we have observed a very open, porous particle structure (Zhang et al., 2016a). This, we believe, leads to a greater hydrodynamic effect on the viscosity than MPWPC and PDWPC's A and B, even though the latter apparently have stronger interactions in solution. Therefore, in PDWPC-C and D solutions hydrodynamic interactions must be dominating over other types of inter-particle interaction. The aggregated protein products also differ from WPC solutions in terms of the relative magnitude of the *G*' and *G*''. For MPWPC, and PDWPC's A and B the *G*'' is higher than *G*' at all protein concentrations, suggesting that the particle solutions dissipate the applied strain mainly through viscous flow rather than storing the applied strain as elastic energy. This is the case also for PDWPC-C and D at the lower solution concentrations (14% and below for both), with a

225 change to elastic response (*G*') dominating over viscous flow (*G*") at higher
226 concentrations.
227 The concentration dependence of the constant plateau value of *G'* in the linear
228 viscoelastic region for the different protein solutions is shown in Figure 2. This
229 figure shows that for WPC the *G*' in the LVER is independent of protein content
230 which was consistent with the findings of Lizarraga et al. (Lizarraga, De Piante
231 Vicin, González, Rubiolo & Santiago, 2006). We also observed the same
232 independence of shear viscosity on protein content for WPC at low shear rates
233 in our previous paper (Zhang et al., 2016b). The aggregated protein products
234 all show a weak dependence of *G*' on concentration at the lower protein
235 concentrations. As the protein concentration increases the elastic modulus *G'*
236 starts to increase at a greater rate and follow a power law dependence, i.e. *G*'
237 ~ c^m where c is the protein concentration and m is the slope of the linear best
238 fit lines in Figure 2. These power law trends at high protein concentrations
239 suggest increased interactions between protein molecules. It was also found
240 that the concentration dependence, i.e. the values of m, as shown in Table 1,
241 of MPWPC and PDWPC-A and PDWPC-B were much smaller than those of
242 PDWPC-C and PDWPC-D. This suggests weaker structure formed by the
243 former ones, while much stronger aggregates, such as network or physical gel
244 existed in the latter ones (Xu et al., 2013). Others have observed a power law
245 relationship between elastic modulus and concentration for both polymer
246 solutions and protein gels. The value of the exponent m varies between protein

247 types. Ferry (1948, 1980) found a value of m=2 for gelatin with higher values in
248 the range m = 2-7 found for other protein gels (Bikbow, Grinberg, Antonov,
249 Tolstoguzov & Schmandke, 1979; Clark, 1989; Kohyama, et al. 1992; Tokita et
250 al. 1985; Vreeker et al. 1992). This includes m= 5 for soybean globulins
251 (Bikbow et al. 1979), m = 3.4 for soybean 11S globulin (Kohyama & Nishinari,
252 1992), m = 4 for casein (Tokita, Niki & Hikichi, 1985), and m = 4.1 for whey
253 protein isolate (Vreeker, Hoekstra, Den Boer & Agterof, 1992). From Table 1 it
254 can be seen that the MPWPC and PDWPC-A and PDWPC-B solutions studied
255 here fall within this range of m=2-7, but PDWPC-C and PDWPC-D have higher
256 values of m suggesting the latter form gels that are more strongly associated in
257 solution than corresponding heat set WPC gels. Also presented in Table 1 is
258 the crossover concentration, at which the G' starts to increase rapidly in Figure
259 2. Presumably, this crossover concentration corresponds to a change in the
260 interaction between the aggregated protein particles, and may correspond to a
261 concentration where hydrodynamic interactions between the particles become
262 important. We also observed a crossover concentration for the equilibrium
263 shear viscosity (Zhang et al. 2016b) at high (100 s^{-1}) and low (0.001 s^{-1}) shear
264 rates, which occurred at similar concentrations.
265
266 To probe the oscillatory response of the solutions of the aggregated protein
267 samples further, frequency sweep measurements were employed. These
268 measurements were carried out for solutions of concentration above 14% w/w.

In Figures 3-7 frequency sweeps and Cox-Merz plots of different protein solutions are shown. Frequency sweeps determine how the material responds to a constant deformation applied over different timescales. Since the time over which the oscillation is applied is equal to the reciprocal of the frequency, low frequency measurements correspond to long time (slow) deformation, and high frequencies to short time (rapid) deformations. The frequency (ω) dependence of G' and G" can give an indication of the type of structure present in the solution. At strains in the LVER region, the frequency sweep can be classified into three general types. Weak gels, such as entangled polymer solutions exhibit a strong dependence of G' with ω, and can exhibit a crossover of the G' and G" curves indicating a change between solid-like behaviour and liquid-like behaviour. Strong, cross-linked gels have only weak dependence of G' on ω and G' always greater than G". In between these two types are the physical gels with some frequency dependence of G' but no G'/G" crossover. Based on these criteria we can classify the gel behaviour of our aggregated whey protein products. For MPWPC, it is found that *G*' is always larger than G" at all protein concentrations studied (Figure 3). Furthermore, the G' has a relatively weak dependence on ω. This suggests the formation of a solid-like physical gel of the microparticulated proteins. PDWPC's A and B (Figures 4 & 5) show a different type of behaviour. Both show a greater dependence of G' on ω than MPWPC. In addition, for PDWPC-A the G" is always higher than G', other than at 21% (w/w) where the two

moduli are virtually the same at all frequencies. Similarly, for PDWPC-B the G'' is higher than G' up to 21% protein, at which concentration G' now dominates over G''. This behaviour is more characteristic of a weak gel network. With PDWPC-C and PDWPC-D the type of gel formed depends on concentration (Figures 6 & 7). At 14% both PDWPC-C and PDWPC-D form a weak gel (relatively strong dependence of G' on ω, with some evidence of a G'/G'' crossover), but formation of physical gels at higher concentrations where G' is always significantly higher than G''.

The Cox-Merz rule is an empirical observation (Cox & Merz, 1958) that for isotropic fluids there is direct correspondence between the complex viscosity measured in a frequency sweep, and the shear viscosity measured as a function of shear rate. Young (2015) states that this means that a fluid that is predominantly a liquid (Newtonian) will obey the Cox-Merz rule, whilst those that are elastic, or Non-Newtonian fluids will not. Thus, adherence (or otherwise) to the Cox-Merz rule can be used to assess further the structure in a system.

Plots of complex viscosity and shear viscosity for the MPWPC solutions (Figure 3) show deviation from the Cox-Merz rule at all concentrations, with complex viscosity higher than shear viscosity at all shear rates and frequencies ω. Such behaviour suggests that the solid-like structure formed in these solutions is susceptible to disruption at large strains (Ikeda & Nishinari, 2001a; Lizarraga et al., 2006). Renard *et al.* (Renard, Robert, Faucheron &

313 Sanchez, 1999) observed similar phenomenon in MPWPC and suggested that
314 it was due to flocculation of the microparticulated proteins at low shear
315 stresses and disruption of these flocs by large shear stresses and strains.
316 The complex viscosity of PDWPC-A solutions (Figure 4) was found to be close
317 to the shear viscosity at concentrations of 14% and 16%, indicating that these
318 solutions followed the Cox-Merz rule and thus, aggregation of proteins was
319 absent or was weak. This would indicate weak interparticle forces and thus
320 fragile aggregates of proteins existing in these solutions. At 18% and 21%
321 solutions of these proteins the complex viscosity is always higher than the
322 shear viscosity suggesting the occurrence of strong interparticle forces and
323 formation of strong aggregates of protein molecules in these solutions.. The
324 Cox-Merz behaviour for PDWPC-B is also complex (Figure 5). At all
325 concentrations deviation from Cox-Merz behaviour is seen at lower shear
326 rate/ω, but at higher shear rate/ω convergence of complex and shear viscosity
327 is observed, suggesting solid-like behaviour over long time scale deformations
328 and liquid-like behaviour at short timescale deformations.
329 Adherence to the empirical Cox-Merz rule was observed only for the 14% and
330 16% solutions of PDWPC-C and PDWPC-D, although as discussed for
331 PDWPC-A and PDWPC-B there was some divergence of shear viscosity from
332 complex viscosity at low shear rates/ω. This would indicate weak interparticle
333 forces and thus fragile aggregates of proteins existing in these solutions. At
334 18% and 21% solutions of these proteins the complex viscosity is always

335 higher than the shear viscosity suggesting the occurrence of strong
336 interparticle forces and formation of strong aggregates of protein molecules in
337 these solutions. Large increases were observed in G', G'', and η^* for the
338 PDWPC-C and PDWPD solutions above 18%, which were much larger than
339 those of MPWPC, PDWPC-B and PDWPC-B at the same concentrations,
340 suggesting a strong network or physical gel formed by the aggregated protein
341 particles.
342 The structure of the MPWPC and PDWPC solutions can be further probed by
343 following the frequency dependence of G' and G''. Close to the gelation point
344 of branched polymers both G' and G'' follow the scaling rule G' or G'' ~ ω^n and
345 the complex viscosity $\eta^* \sim \omega^{n-1}$ (Muthukumar, 1989). Several attempts have
346 been made to relate the exponent n to the structure or fractal dimension of
347 gels. The fractal dimension is often used to characterise the structure of
348 aggregated or gelled systems (Hagiwara, Kumagai, Matsunaga & Nakamura,
349 1997). Computer simulations have been used to establish the theoretical
350 fractal dimension (d_f) value for two limiting forms of aggregation – diffusion
351 limited (DLA) and reaction limited (RLA) (Witten & Sander, 1981; Weitz,
352 Huang, Lin & Sung, 1985). In DLA, particles collide and stick together in a rigid
353 structure that does not rearrange with time. Aggregates of gels formed from
354 this type of aggregation are open and diffuse, and are characterised by fractal
355 dimensions around 1.8. In RLA, on the other hand, particles aggregate more
356 loosely, and the aggregates formed are able to rearrange over time.

Aggregates formed via RLA have a more compact, dense structure with a fractal dimension of around 2.1. The value of d_f =1.8 is the lower practical limit for 3-dimensional systems. The upper limit for d_f is the Euclidian dimensionality of the system, i.e. 3 for 3-D systems and 2 for 2-D systems. Percolation theory predicts that (Stauffer, 1985),

$$n = \frac{d}{d_f + 2} \qquad (1)$$

where d is the Euclidian dimensionality of the system (d=3 in this case) and d_f the fractal dimension. For percolation theory, d_f = 3 corresponds to n=0.6, and we would expect fractal gels to have n>0.6. Muthkumar (1989) however, found very small values of n for polymer solutions close to the gel point, which would give unrealistically high (>3) d_f values based on percolation theory (eqn. 1). Doi & Edwards, (1986) explained this as being due to the screening of the excluded volume effects due to high concentration of polymers. Muthkumar (1989) derived an alternative relationship for n, taking account of screening excluded volume effects, as,

$$n = \frac{d\left(d + 2 - 2d_f\right)}{2\left(d + 2 - d_f\right)} \qquad (2)$$

The value of n for the scaling of G', G'' and η^* for the MPWPC and PDWPC's was calculated and is presented as an average of the three in Table 2 along with the calculated average d_f. From the discussion above, we would expect d_f to fall approximately within the range 1.8 - 2.1. Values of d_f lower than 1.8 have

been observed for fibrillar aggregates formed by polyvinyl chloride (Dahmani, Fazel, Munch & Guenet, 1997) where the aggregate takes a more linear form. Similar fibrillar gels are not unknown for proteins (Nicolai & Durand, 2013). For protein gels, d_f higher than 2.1 but lower than 3 is not uncommon at higher protein concentrations as the packing density of the molecules can be high, and restructuring of the gel after formation is common (Eleya, Ko & Gunasekaran, 2004). Percolation theory assumes that polymer solutions show fractal properties close to the percolation threshold, i.e. the concentration at which a percolating network just forms. When fractal dimension is calculated using percolation theory (eqn. 1) for all samples, a wide range of d_f values are found. For MPWPC, the d_f values are all >> 3. This suggests that at 14% MPWPC and above we are above the percolation threshold and so percolation theory does not hold. If we look at the crossover concentration (m) in Table 1, we see that this is about 12%. Therefore it is not unreasonable to suggest that the crossover concentration in Figure 2 might correspond to the percolation threshold, explaining why d_f is indeterminate above the crossover concentration. A similar result is observed for PDWPC-C and PDWPC-D where d_f calculated from eqn.1 are large at all concentrations of 14% and above, suggesting the solutions are above the percolation threshold. Again, from Table 1 we see that the crossover concentration for these two protein products is 12%, i.e. below the concentrations where d_f is calculated in Table 2. For PDWPC-A and PDWPC-B the results are more complex. For PDWPC-A

401 the crossover concentration in Table 1 is 16%. At 16% PDWPC-A solutions
402 and below, the d_f calculated from percolation theory is less than 1.8, and above
403 16% it is high and close to 3. This again suggests that percolation theory does
404 not hold for PDWPC-A at these concentrations and that the percolation
405 threshold is between 16-18%. Similarly, for PDWPC-B the observed crossover
406 concentration is also 16%. In this case, the 16% solution of PDWPC-B gives a
407 sensible value for d_f calculated from eqn. 1, but below this concentration $d_f <$
408 1.8 and above 16% it is > 3. These results suggest that the percolation
409 threshold for PDWPC-B solutions is close to 16%, thus adding more weight to
410 the supposition that the crossover concentration is equivalent to the
411 percolation threshold.
412 If screening of the excluded volume contributions is assumed in these
413 solutions, and Muthkumar's equation for calculating d_f is used (Muthkumar,
414 1989) more sensible value of fractal dimension are found at concentrations
415 close to and above the crossover concentration. For MPWPC eqn. 2 yields d_f
416 values of 2.1-2.2 at all concentrations, values that are close to that for RLA.
417 Winter and Chambon (1986) and Vilgis and Winter (1988) proposed the power
418 law exponent and the fractal dimension for a critical gel where the network
419 expanded to infinite are 0.50 and 2.0. This suggests that MPWPC solutions
420 form a fractal network at all concentrations studied. This would be consistent
421 with the observation from our previous work that MPWPC solutions contain
422 large flocs of smaller particles that would show fractal behaviour over a wide

423 range of length scales and concentrations both above and below the crossover
424 concentration. For PDWPC-A and PDWPC-B eqn. 2 predicts low d_f values
425 below the crossover concentration, and value around 1.6-1.7 above this.
426 Below the crossover concentration, it seems likely that open extended
427 structures form by association between PDWPC-A and PDWPC-B particles.
428 PDWPC-A and PDWPC-B (and MPWPC) also showed strong strain overshoot
429 indicating strong interactions between the particles, and this may lead to the
430 tendency for more extended aggregates below the crossover concentration.
431 For PDWPC-C and PDWPC-D the solutions exhibit fractal structures that
432 become more compact (d_f increases from 1.6 to 2.3 for PDWPC-C and from
433 1.9 to 2.3 for PDWPC-D) with increasing concentration. This suggests that a
434 more rubber-like (Macosko, 1994; Rao, 2007) and denser structure (Ikeda &
435 Nishinari, 2001a; Vilgis & Winter, 1988) forms at higher protein concentrations.
436 Invalidity of the power law was also found at low frequencies for both of
437 PDWPC-C and PDWPC-D gels, thus indicating that the network of the proteins
438 had a finite length scale. According to the interpretation of mechanical spectra
439 by Vilgis & Winter (1988) the frequency, ω, of the oscillations can be related to
440 the length scale, L of the fractal network as $\omega^{-1} \propto t \propto L^d$, where t is the time
441 for relaxation and d is determined by diffusion of the polymers. Consequently,
442 a limiting characteristic frequency, which defines the largest length scale of the
443 gel, can be obtained. This is defined by the frequency at which linear scaling of
444 G'/G" with ω stops/starts. From Figures 6 and 7 the characteristic frequencies

445 for PDWPC-C and PDWPC-D were found to be the same at 0.02 rad/s,
446 indicating the same length scale of the self-similar network of these two
447 denatured protein solutions.

448 Many foods systems are acidic in pH, a situation that leads to inhibition of
449 growth of spoilage bacteria. The pH also has an effect on protein as they
450 contain acidic and basic amino acid groups, and at a certain pH, the isolectric
451 point or pI, the net charge they have is neutral. Therefore, the pH might be
452 expected to influence the interactions and structure of solutions of proteins and
453 partially denatured proteins. Since WPC is a mixture of proteins containing
454 mainly β-lactoglobulin as well as α-lactalbumin and bovine serum albumin
455 (BSA), each of which have different isoelectric points (Bryant & McClements,
456 1998; de Wit & Klarenbeek, 1984; Kinsella & Whitehead, 1988), a definition of
457 the isoelectric point (pI) of WPC is complicated. Marinova *et al.* (2009)
458 proposed a concept of 'effective' pI for WPC and suggested that it is about 4.2
459 where the ζ-potential of the proteins is zero. In previous work we have
460 measured the pI of Lacprodan 87 as 4.5. To test the effect of acidic pH on the
461 MPWPC and PDWPC solutions, acetic acid was added to lower the pH to 4.5
462 to approach the pI of proteins and decrease the charges on the protein
463 surface.

464 The effect of lowering the pH on G' and G'' of the PDWPCs at a concentration
465 of 21% (w/w) are illustrated in Figure 8. Both G' and G'' increased for all
466 samples when the pH was reduced to the pI, suggesting strengthening of the

structure and increased interaction between protein particles, most likely from a reduction in electrostatic repulsion (Elofsson, Dejmek, Paulsson & Burling, 1997; Goodwin & Hughes, 2008; Ikeda, Foegeding & Hagiwara, 1999). For MPWPC, G' is always higher than G'' at pH 4.5, and the dependence of G' and G'' on ω is relatively weak as it was at the higher pH (Figure 3) indicating that the solid-like physical gel structure of the MPWPC is maintained at pH 4.5. Cox-Merz plots for MPWPC at pH 4.5 show that the solutions do not adhere to the Cox-Merz rule, the same as at the higher pH, with the complex viscosity higher than the shear viscosity at all frequencies. The lack of adherence to the Cox-Merz rule indicates stronger interfloc forces, and thus, stiffer aggregating structure of MPWPC at low pH (Macosko, 1994; Rao, 2007). Both the complex and shear viscosity at pH 4.5 are higher than that at the higher pH.

For PDWPC A and PDWPC B there is a weaker dependence of G' on ω at pH 4.5, and G' is always greater than G'' suggesting a strengthening of the gel structure (Figure 8). The Cox-Merz rule solutions is also not followed for PDWPC A and PDWPC B at pH 4.5 (Figure 9), whereas the rule is followed at higher pH. More obvious violations of the Cox-Merz rule are seen for the acidic solutions compared to those at natural pH of the protein solution, where the violations are only present at low frequencies and shear rates. Therefore, strain- susceptible structure of protein aggregates could be hypothesised to form in the acidic solutions (Ikeda & Nishinari, 2001a).The complex viscosity at pH 4.5 is always higher than at the higher pH for both PDWPC A and B, but the

shear viscosity shows unusual behaviour. For both PDWPC A and B, the shear viscosity at pH 4.5 at low shear rates is higher than at the higher pH (Figure 9). However, as shear rate increases the shear viscosity for PDWPC A becomes the same as that at the higher pH, but for PDWPC B there is a crossover and shear viscosity at higher shear rates is lower for pH 4.5. A possible explanation for this is that as well as altering the interactions between particles, a lower pH also causes changes to the internal structure of the particles for PDWPC A and B. It is possible that the particle swells at low pH and become more sensitive to disruption due to high shear rates. At pH 4.5 the proteins will be close to the isoelectric point, and the proteins may flocculate in the absence of electrostatic repulsion. These flocs are likely to be shear sensitive, and will break up at higher shear rates leading to shear thinning behaviour.

For both PDWPC-C and PDWPC-D both G' and G'' increase at the lower pH. PDWPC-C and PDWPC-D exhibited larger complex viscosity at pH of 4.5 than at natural pH, while there was no change in the shear viscosity, except at low shear rates, where aggregates of proteins were supposed to be formed and incompletely disrupted (Renard et al., 1999). Such observations suggest that the gel formed by polymeric denatured protein is strengthened at pH 4.5 since the electrostatic repulsions were reduced, but the flowing units remained the same as those at natural pH when the gel structure was disrupted by large strains.

511 A power law was found to hold for the solutions of all PDWPC solutions at pH
512 4.5, but with smaller values of n (Table 2) and higher fractal dimensions. The
513 lower values of n (slope) of the G' vs ω graphs (Figure 8) at pH 4.6 indicate a
514 reduced frequency dependence of G' and reveal a more solid-like behaviour of
515 the solutions (Goodwin & Hughes, 2008; Macosko, 1994; Rao, 2007). These
516 more solid-like properties could be due to the denser packing of the proteins in
517 the solutions.
518 Since there is no net charge on the protein surfaces at pI, hydrophobic
519 interactions and van der Waals attractions will predominate the electrostatic
520 repulsions between protein molecules and flocs, which causes the further
521 aggregation and flocculation of proteins (Ikeda et al., 1999; Verheul, Pedersen,
522 Roefs, & de Kruif, 1999). Increased hydrodynamic interactions between
523 protein flocs with larger volumes at pH of 4.5 would account for the increases
524 in viscosity of MPWPC and PDWPC solutions.
525 The phenomenon observed here is similar to cold gelation of whey proteins
526 observed by other researchers (Bryant & McClements, 1998). These are
527 produced by heating under similar conditions to the PDWPC's (pH close to 7,
528 temperatures above 70 ^{o}C) at low salt and low protein concentrations to
529 prevent protein aggregation and gelation (Barbut & Foegeding, 1993). The
530 solutions of these proteins can be induced to thicken or gel by addition of salts
531 (Barbut & Foegeding, 1993), or reduction of pH (Alting et al, 2004). The critical
532 difference between previously reported cold-gelling WPCs and the PDWPC's

533 studied here is that higher protein concentrations (20-26% total solids) can be
534 processed compared to cold-gelling WPCs. This is due to monitoring and
535 control of the denaturation process that ensures over aggregation and gelation
536 does not occur. This makes the process economically feasible for production
537 of PDWPC's.

538 **Conclusion**

539 The viscoelastic properties of solutions of PDWPC aggregates exhibit a
540 complex dependence on the protein concentrations and microstructure of the
541 particles. The solution behaviour of PDWPC's differs from and is more
542 complex than both WPC and MPWPC, differences that can be explained by
543 structural differences between MPWPC and PDWPC protein particles that lead
544 to differences in the way they interact. Aggregated particles with open,
545 fibril/tubule-like structures, such as found in PDWPC-D have more solid like
546 behaviour, whilst compact aggregates such as found in PDWPC-A and –B
547 have a more liquid-like structure. The rheological behaviour of the PDWPCs is
548 strongly concentration and frequency dependent, with changes between more-
549 liquid like and more solid-like behaviour occurring as protein concentration
550 increases, and with a change from long timescale (low frequency) to short
551 timescale (high frequency) deformations. The PDWPCs exhibit a complex
552 rheological behaviour which suggests they could be versatile thickening,
553 texturizing and fat replacement ingredients that will offer food manufacturers

554 greater control over the texture of formulated fluid foods.

555

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Table Legends

Table 1 – Power law exponent, m, for the concentration dependence of G' for different protein samples.

Table 2 – Power law exponent, n, from the scaling of *G*', *G*'' or η^* with frequency (ω) and the fractal dimension percolation theory (eqn. 1) or eqn. 2.

Figure Legends

Figure 1 - Strain dependence measured at 1 Hz of elastic, G' (●), and viscous, G'' (○) moduli of protein product solution at a protein concentration of 21% (w/w).

Figure 2 Log-log plot of the concentration dependence of G' in linear viscoelastic region for WPC, MPWPC, PDWPC-A, PDWPC-B, PDWPC-C, PDWPC-D solutions

Figure 3 – Frequency sweep and Cox-Merz plots of MPWPC solutions.

Figure 4 - Frequency sweep and Cox-Merz plots of PDWPC-A solutions.

Figure 5 - Frequency sweep and Cox-Merz plots of PDWPC-B solutions.

Figure 6 - Frequency sweep and Cox-Merz plots of PDWPC-C solutions.

Figure 7 - Frequency sweep and Cox-Merz plots of PDWPC-D solutions.

Figure 8 - Comparison of the frequency dependence of *G*' and *G*'' for PDWPC solutions (21% w/w) at pH4.5 and at the natural pH of the solution.

Figure 9 - Comparison of the shear rate dependence of shear viscosity (η) and frequency dependence of complex viscosity (η^*) for PDWPC solutions (21% w/w) at pH4.5 and at the natural pH of the solution.

738 **Table 1**

739

	Concentration dependence (*m*)		Crossover concentration (C^*) (%)
	Above C^*	Below C^*	
WPC	-	-	-
MPWPC	0.59	5.68	12
PDWPC-A	0.47	5.90	16
PDWPC-B	0.60	6.05	16
PDWPC-C	0.40	12.04	12
PDWPC-D	0.47	12.90	12

740

741 **Table 2**

Protein Product	Mean n	d_f Percolation theory (Stauffer, 1989)	d_f Screened excluded volume (Muthkumar, 1989)
MPWPC			
14%	0.20 ± 0.10	14.6 ± 4.8	2.2 ± 0.1
16%	0.20 ± 0.00	13.0 ± 3.0	2.2 ± 0.1
18%	0.20 ± 0.10	13.8 ± 5.4	2.2 ± 0.1
21%	0.30 ± 0.10	9.3 ± 2.7	2.1 ± 0.1
pH4.5 (21%)	0.19 ± 0.03	-	2.3 ± 0.0
PDWPC-A			
14%	1.18 ± 0.34	1.0 ± 0.7	1.0 ± 0.4
16%	0.95 ± 0.22	1.3 ± 0.7	1.1 ± 0.4
18%	0.62 ± 0.02	2.8 ± 0.1	1.6 ± 0.0
21%	0.60 ± 0.02	3.0 ± 0.2	1.7 ± 0.0
pH4.5 (21%)	0.27 ± 0.03	-	2.1 ± 0.0
PDWPC-B			
14%	0.85 ± 0.08	1.5 ± 0.3	1.3 ± 0.1
16%	0.73 ± 0.06	2.1 ± 0.3	1.5 ± 0.1
18%	0.57 ± 0.03	3.3 ± 0.3	1.7 ± 0.0
21%	0.36 ± 0.04	6.5 ± 0.9	2.0 ± 0.1
pH4.5 (21%)	0.14 ± 0.01	-	2.3 ± 0.0
PDWPC-C			
14%	0.47 ± 0.01	4.4 ± 0.1	1.9 ± 0.0
16%	0.31 ± 0.02	7.6 ± 0.5	2.1 ± 0.0
18%	0.17 ± 0.00	15.6 ± 0.0	2.3 ± 0.0
21%	0.19 ± 0.01	14.1 ± 0.5	2.3 ± 0.0
pH4.5 (21%)	0.13 ± 0.01	-	2.3 ± 0.0
PDWPC-D			
14%	0.63 ± 0.02	2.8 ± 0.2	1.6 ± 0.0
16%	0.33 ± 0.04	7.2 ± 1.0	2.1 ± 0.1
18%	0.27 ± 0.02	9.3 ± 0.9	2.2 ± 0.0
21%	0.19 ± 0.02	13.6 ± 1.2	2.3 ± 0.0
pH4.5 (21%)	0.16 ± 0.01	-	2.3 ± 0.0

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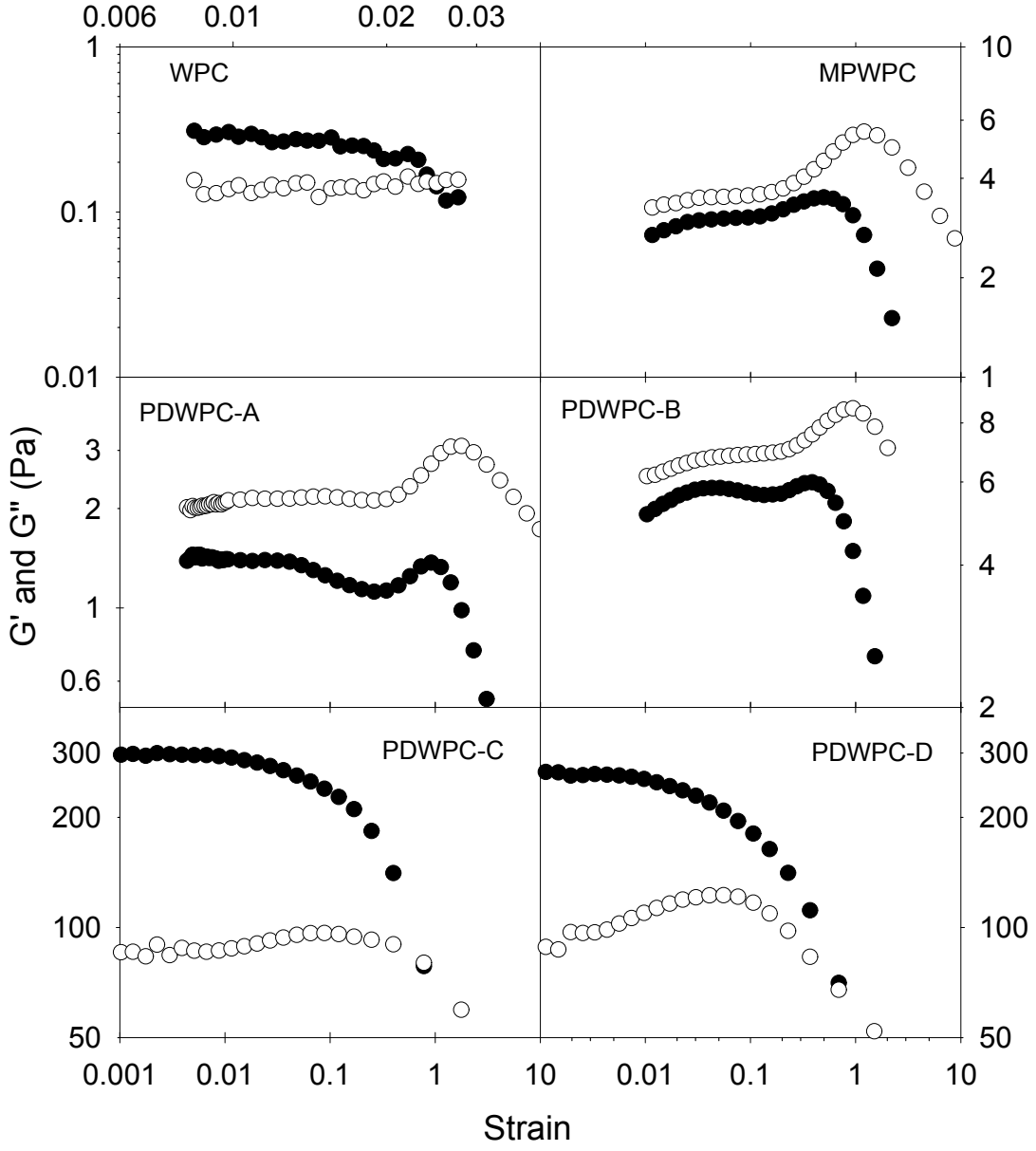

Figure 1

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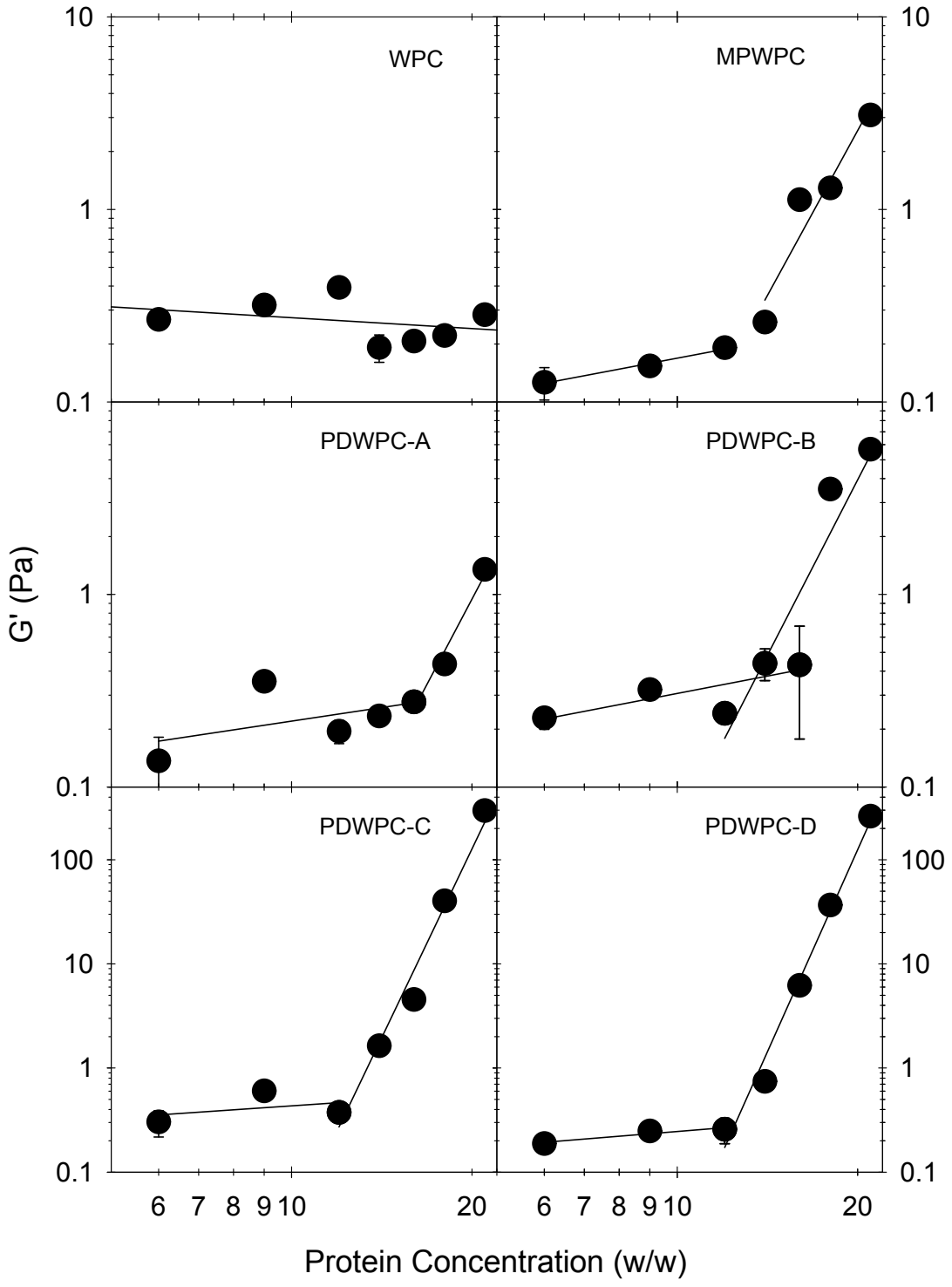

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757
758 **Figure 2**.

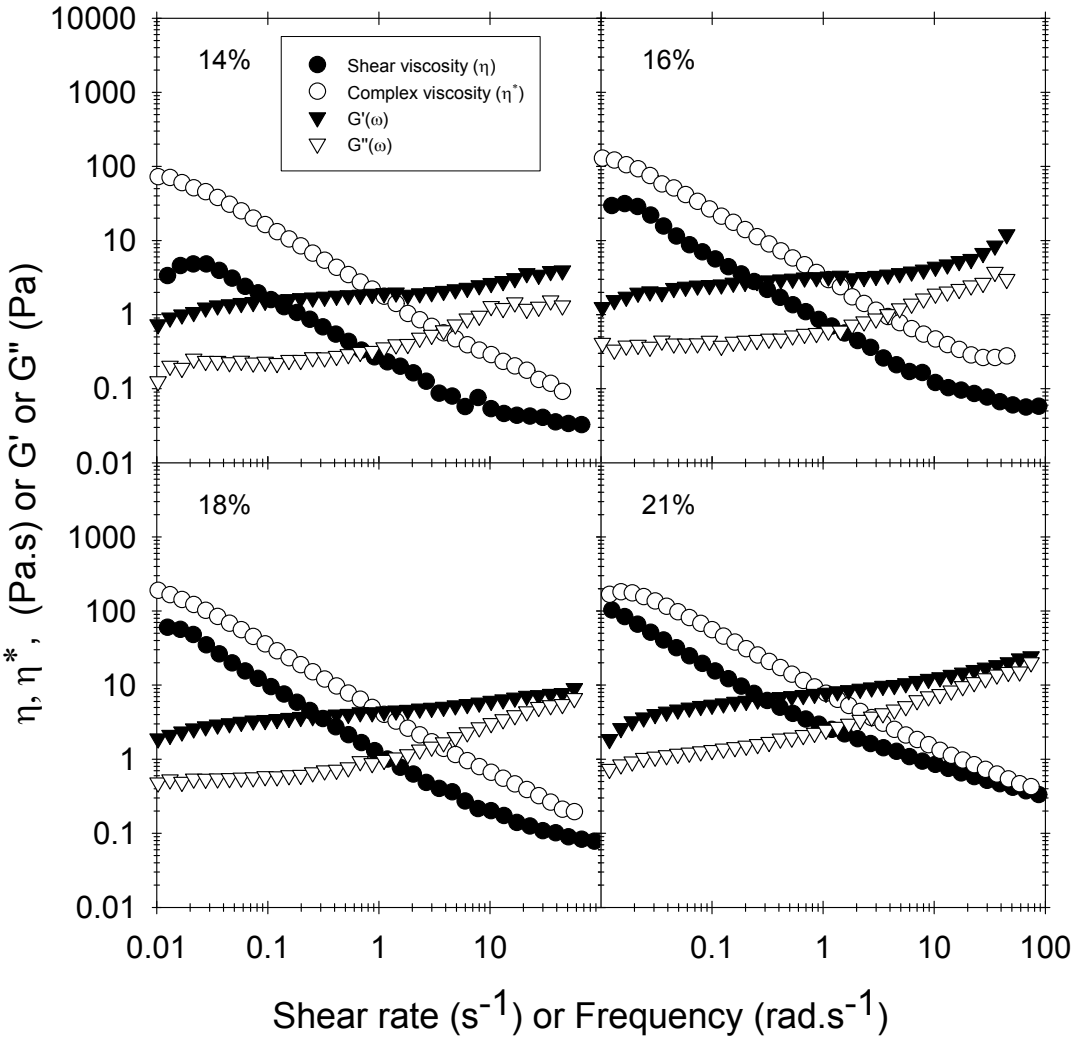
14%
16%
18%
21%
Shear viscosity (η)
Complex viscosity (η*)
G'(ω)
G"(ω)
η, η*, (Pa.s) or G' or G" (Pa)
Shear rate (s-1) or Frequency (rad.s-1)
10000
1000
100
10
1
0.1
0.01
0.01
0.1
1
10
100

Figure 3

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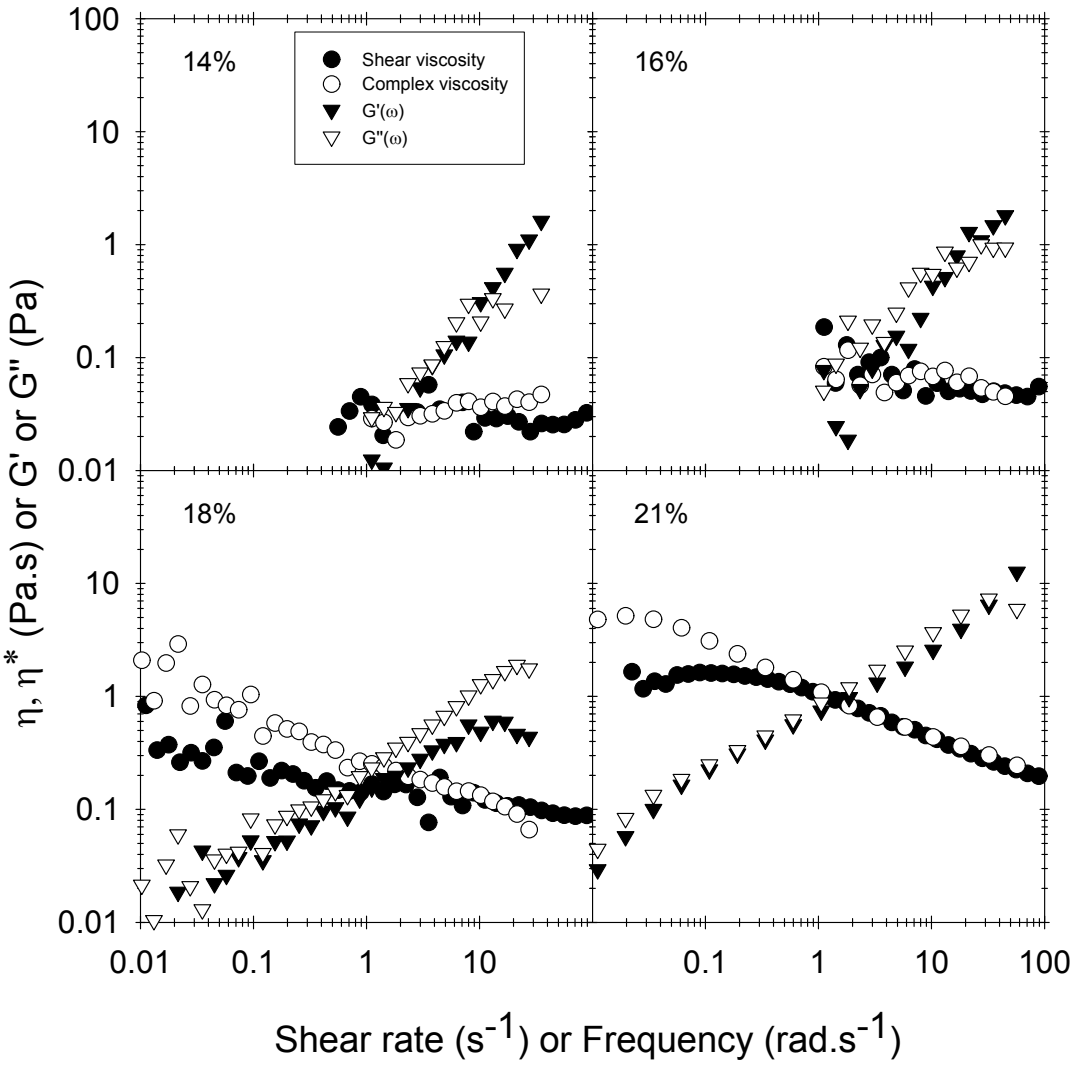

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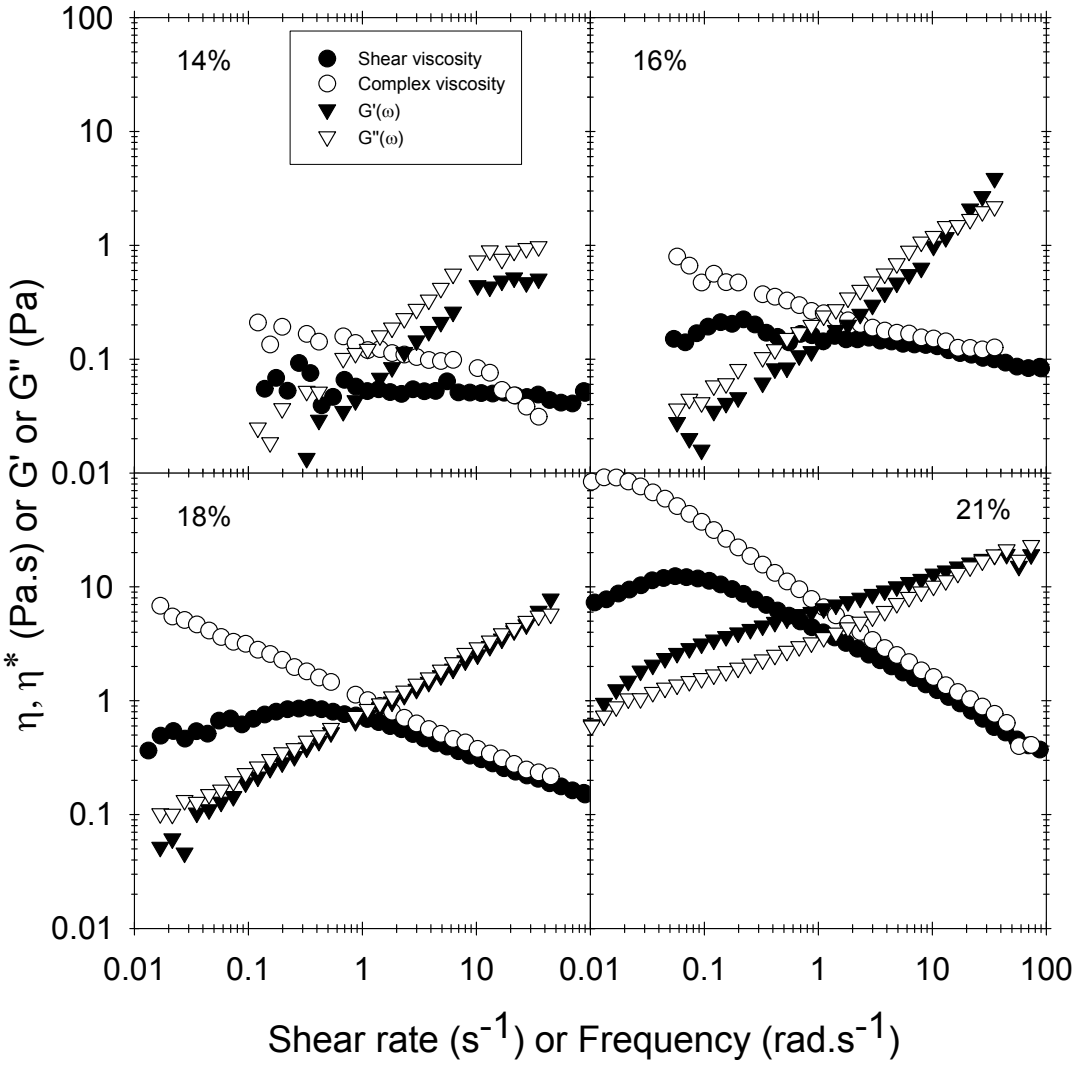
768 **Figure 4**



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771 **Figure 5**

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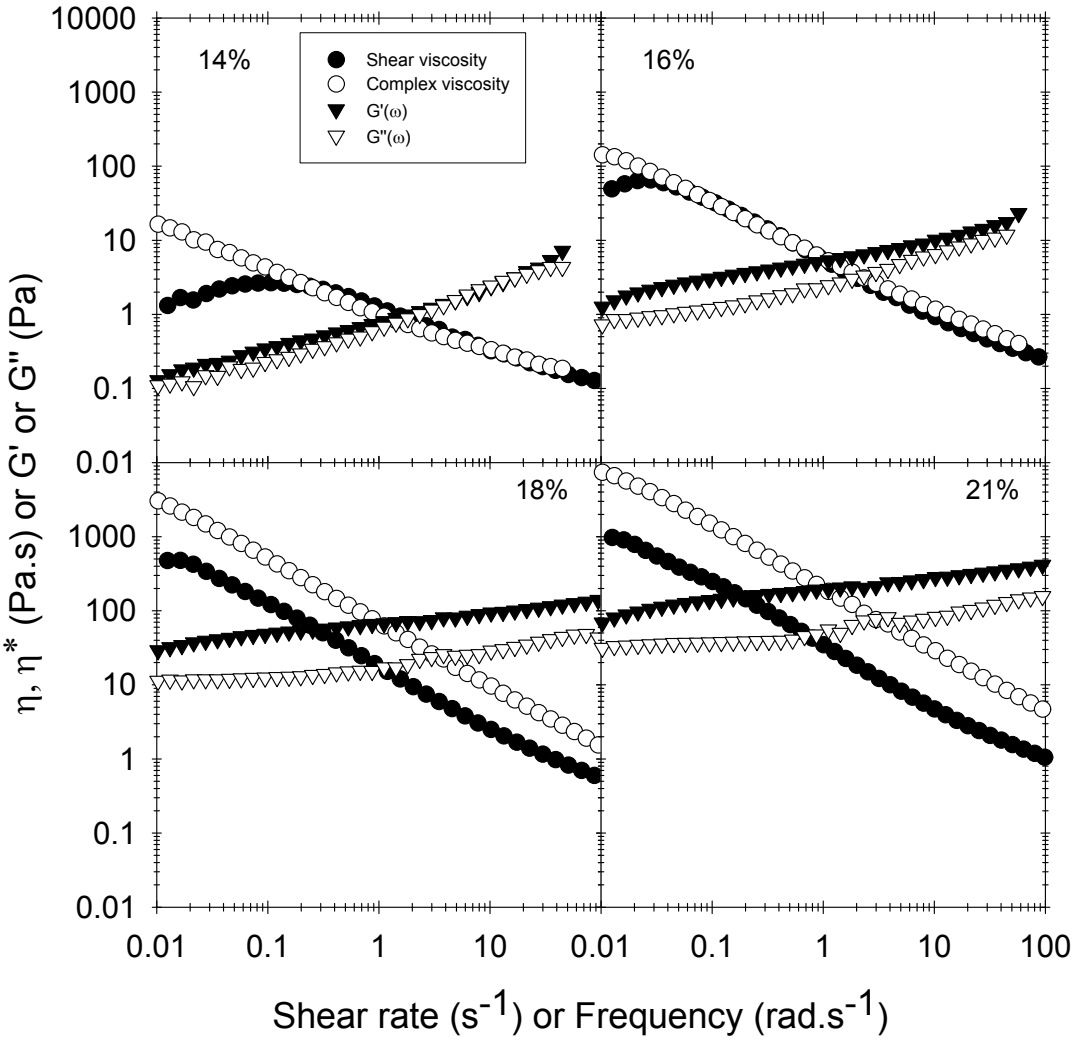

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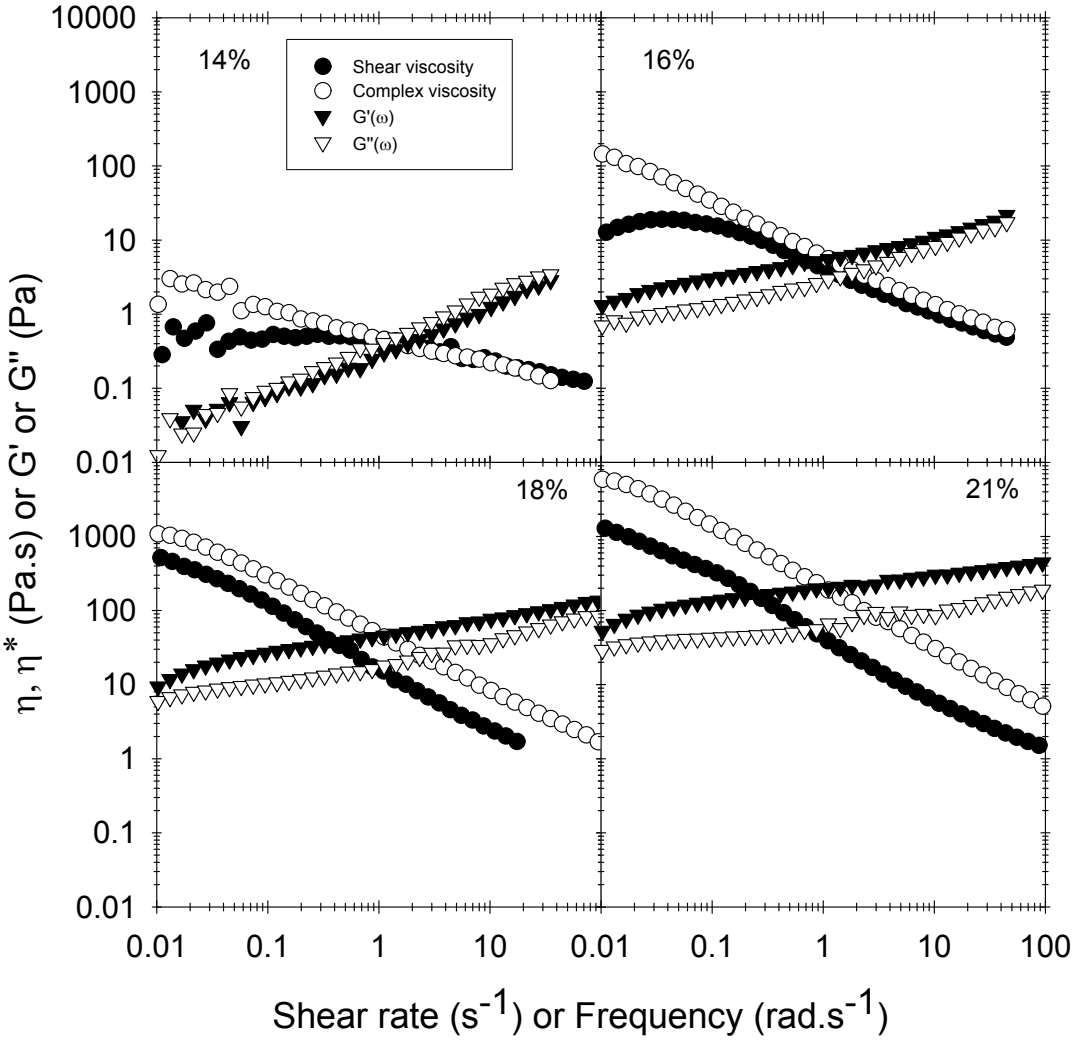
774

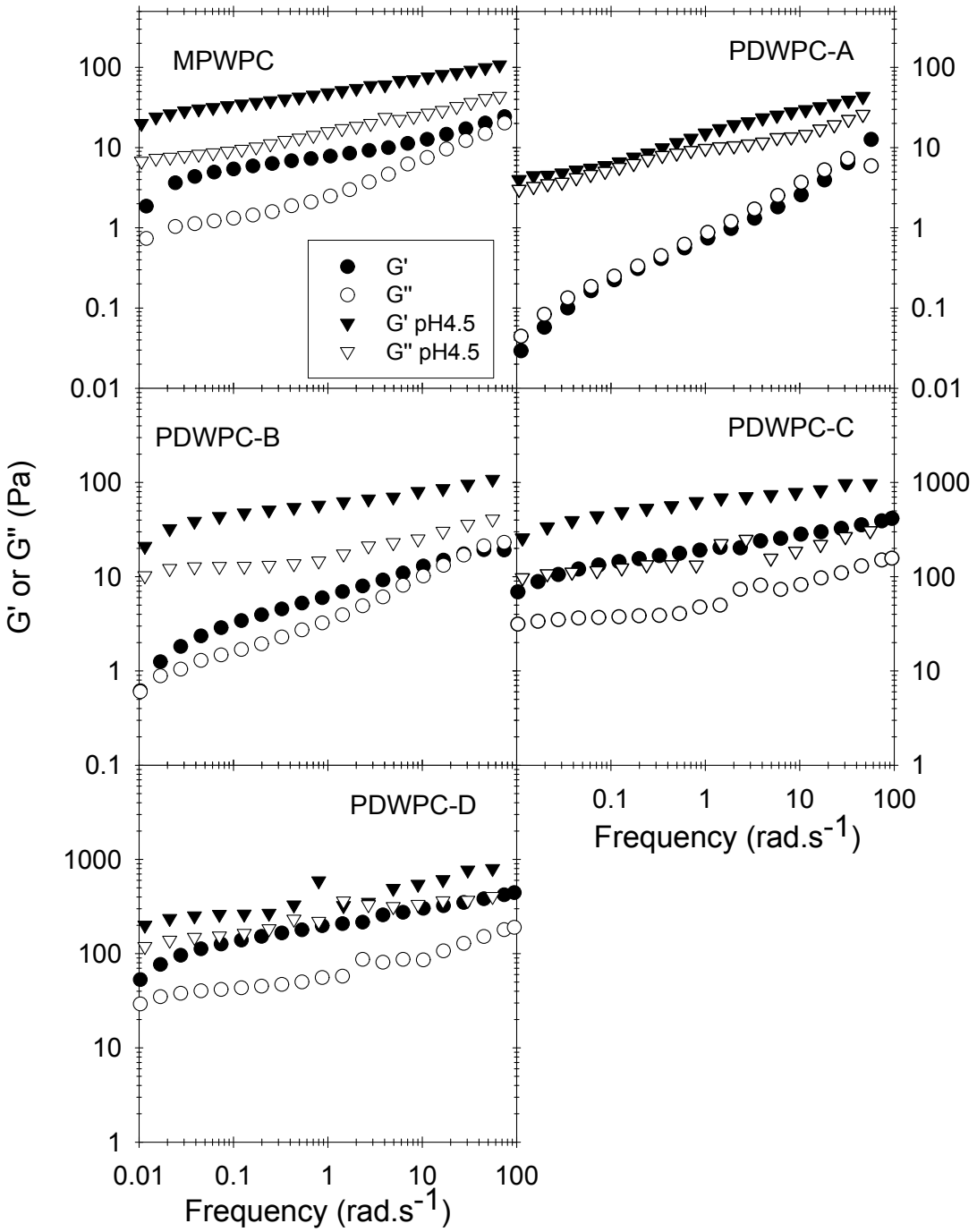
775 **Figure 6**



776

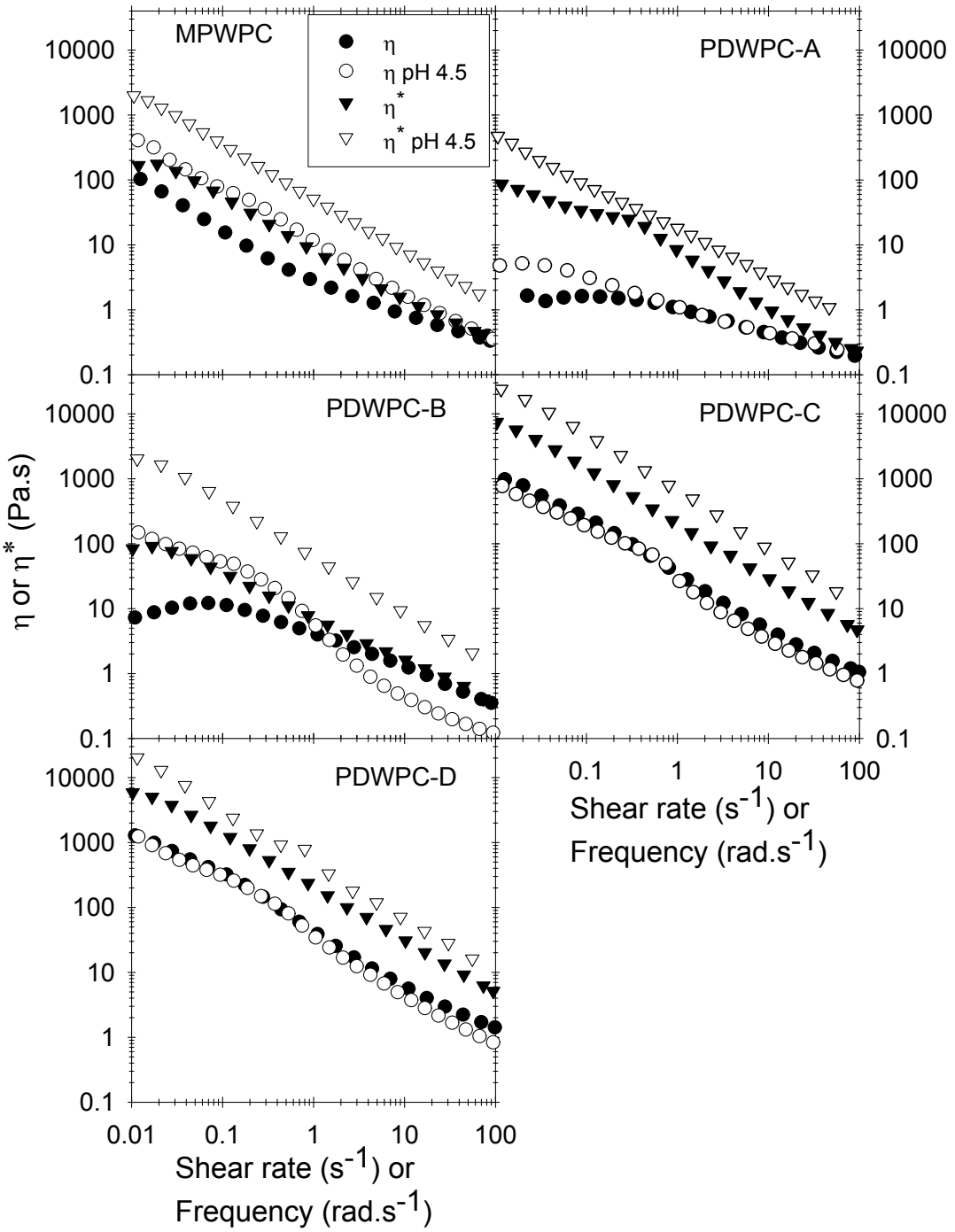
777

778 **Figure 7**



779

780 **Figure 8**



781

782 **Figure 9**

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Highlights

- PDWPCs can be classified based on the viscoelastic properties of their solutions
- PDWPCs with compact particles exhibit strong strain overshoot and form weak gels
- PDWPCs which have open, porous particles show weak strain overshoot
- PDWPCs with open particles form weak gels or strong gels depending on concentration
- All PDWPCs form fractal aggregates in solution